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RESEARCH ARTICLE

Molecular Detection and Evaluation of Some Immunological Parameter on Salmonellosis Patients in Al-Muthanna Province

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Abstract

One hundred samples were collected from human blood depended on age, gender, region, taking medication, and smoking during the extended period from December/ 2015 to July/ 2016 in Al Hussein Teaching Hospital of Al-Muthanna Province. Forty-five samples were positive to Widal test as titer ≥ 1:320. Real Time Polymerase Chain Reaction technique was done on DNA samples extracted from 45 positive Widal test fresh blood samples, that's done by using Flic as diagnostic gene and SYBR Green I dye. The results showed 21 (46.6 %) samples were positive and 24 (53.3 %) were negative. The nitro blue tetrazolium (NBT) test was done to estimate the phagocytic activity in positive Widal test patients. The results of (NBT) test showed a significant increase (P<0.05) in phagocytic activity in the blood of patients who had positive Widal test, compared with the blood of healthy individuals (79.3%) as a positive control. The MTT assay was used for evaluating the lymphocyte transformation index % of peripheral blood leukocytes in positive Widal test patients. The results of MTT assay showed a significant increase (P<0.05) in the lymphocyte transformation index in the lymphocytes of positive Widal test patients, compared with the lymphocytes of healthy individuals (75.6%) as a positive control.

Key words: Human-Salmonella typhi, PCR and Widal.

Introduction

Salmonella is a bacterial genus within the Family Entero bacteriaceae that consists of a large group of genetically similar organisms with the ability to infect a large number of animal hosts [1, 2].The majority of clinical disease in animals and humans is caused by serovars within the Salmonella enteric subspecies and this can range from local gastroenteritis to a fatal disseminated disease. The exact clinical outcome of Salmonella infection depends largely on the individual serovars involved, the infected host species and the immunological status of the individual [1, 2]. Salmonella serovars Typhi and Paratyphoid have a restricted host range and cause systemic disease in humans that can often be fatal [3]. Salmonella enteric is a flagellated, Gram-negative, facultative intracellular bacterial species that is a leading cause of enteric disease in humans and in animal hosts.

Salmonellae are taken up via contaminated food and can infect a broad range of hosts; certain host-adapted serovars can even cause a severe systemic disease. For example, the human restricted S. enteric serovartyphi causes typhoid fever, which affects over 20 million people worldwide and leads to approximately 200,000 deaths per year.

Typhoid fever is a serious health problem in many developing countries. Worldwide, an estimated 17 million persons develop this disease annually. Most of this burden occurs among citizens of low-income countries, particular those in Southeast Asia, Africa, and Latin America, in South Sulawesi, Indonesia [4]. The disease is endemic throughout the region and is the fourth most frequently reported infectious disease in most of its 24 districts. In South-Sulawesi, typhoid is the most important cause of community-acquired septicemia, with a reported incidence rate exceeding 2,500/100,000 in many districts.

Typhoid fever is caused by Salmonella entericaserovar Typhi and is transmitted through the fecal-oral route by consumption of contaminated water and food. The presence of a convalescent patient or a carrier actively shedding the pathogen poses an increased risk for infection. In no endemic areas, disease outbreaks may occur from a unique source of food or carrier [5]. In disease-endemic areas a recent contact with a patient or carrier has been identified as a major risk factor [4, 5] but other risk factors include poverty, low education level, poor hygienic conditions and water supplies, and eating outdoors at food stalls [4, 6].

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Almost half of the treated patients continue to excrete the pathogen one month after the symptoms have disappeared, and approximately 5% still do so five months later [7]. Approximately 3% become carriers and continue to excrete the organism, often lifelong. The carrier stage may also develop after an asymptomatic infection. Molecular detection methods are most suitable to identify pathogens in human excretions because these methods are highly specific and sensitive. In particular the PCR is capable of detecting minute quantities of DNA of specific pathogens through amplification of a defined DNA segment, and discriminating in one reaction between different organisms even if they are closely related.

In combination with the appropriate sample preparation method, PCR can be applied on almost any specimen including whole blood, stool, and urine [8]. Therefore, PCR seems to be suitable to identify those patients actively excreting the organism and to investigate the carrier stage through the specific detection the DNA of S. typhiin blood samples. In this study, we assessed the sensitivity of the real time PCR on blood samples and compared with Widal test and immunological parameters (NBT and MTT).

Materials and Methods

One hundred samples were gathered from blood of patients depended on age, gender, region, taking medication, and smoking individuals suspected with Salmonellosis depending on manifestations of patients, including fever, head torment, drowsiness, sweating, and joint pain during the extended period from December/ 2015 to July/ 2016 in Al-Hussein Teaching Hospital of Al-Muthanna Province.

The Widal test with O antigen (Murex Biotech Ltd., Dart ford, United Kingdom) was performed and interpreted according to routine laboratory procedures. A titer ≥1:320 were considered positive. For preparation of bacterial DNA from blood samples, the bacterial genomic DNA of Salmonella extracted by using (Presto™ Mini g DNA Bacteria Kit, Gene aid. The USA), and done according to company instructions.

Quantitative Real-time Polymerase chain reaction (RT-PCR) (RT-PCR) technique was performed to detect Salmonella. The method was carried out according to method described by [9]. The PCR primers used in our study shown in (Table 2) (Bioneer, Korea). QPCR master mix was set up by utilizing Accu Power TM Green Star Real-Time PCR kit that utilizing SYBER green dye recognition of gene intensification in Real Time PCR system.

Primer		Sequence	Target gene
ST1	F	'5-ACT GCT AAA ACC ACT ACT -3'	flagellin gene (fliC)
ST2	R	'5- TTA ACG CAG TAA AGA GAG -3'	

Table 1: The PCR primer used in current study to diagnosis Salmonellosis.

qPCR master mix	Volume
DNA template	2.5μL
Forward primer (10pmol)	1 μL
Reverse primer (10pmol)	1 μL
Nuclease-free water	15.5 μL
Total	20 μL

Table 2: The components of master mix preparation.

After that, these qPCR master mix segments that specified above AccuPower Green Star qPCR premix standard plate tubes that contain the SYBER green dye and other PCR intensification segments, then the plate blended by Exispin vortex rotator for 3 minutes, then put in the Minopticon Real-Time PCR framework. After that, the qPCR plate was stacked and the accompanying thermo cycler convention in the accompanying.

Nitro blue Tetrazolium (NBT) test was carried out in relation to a method offered by [10] for all patients. NBT was used for evaluation of phagocytic activity percentage. The MTT assay performed according to (MTT cell viability kit, Abnova, USA) a method used for estimation of lymphocytes transformation. The lymphocyte transformation index percentage calculated according to the following equation [11].

Lymphocytes transformation index (%) = $\{(A-B)/B\} \times 100$

A= Treated group; B= Negative control group.

Data were subjected to statistical analysis using SAS program (Statistical Analysis System) version 9.1 (2010). Criteria used in the evaluation of the traditional test in the present study were, according to (Parikh et al., 2008). Two-way ANOVA with Interaction and Least significant differences (LSD) post hoc test was performed (multiple comparisons), to assess significant difference among means. $P<0.05$ was considered statistically significant.

Results & Discussion

Widal test

Widal test is secondary immune reaction depending on binding of antigen and antibody with refer to the subsequent effects in the diagnosis of salmonellosis disease, means the non-reactive antigens , antibodies and the nonspecific reaction. Also these reactions are based on the ability of antibodies to crosslink particulate antigens and some factors effect agglutination involved antigen concentration and antibodies titration must be known (excess antigen give soluble results),diluents must be known and with pH more than 6 or neutral (Nacland PBs),suitable temperature 37,suitable time and the serum must be clear and transparent. Therefore, IgM Abs, because of their larger size, tend to be stronger agglutinins than IgG Abs [13, 14].The study was conducted on suspected patients with salmonellosis infection in Al-Hussein teaching Hospital of Al-Muthanna Province, 100 samples were collected from peripheral human blood, 45 (45%) was positive Widal test and others 55 samples have been discarded. The result show high percentage of salmonellosis infection in Al-Muthanna Province peoples (45%) positive Widal test due to high ratio of false positive result of Widal test. Our result agreed with results obtained by [12] who noted that the resulting Widal result may lack sensitivity and specificity. Also may reflect a population immunologically sensitized by regular subclinical exposure to S. typhi, particularly in a community with endemic typhoid fever [15].

Quantitative Real-time Polymerase chain reaction (qPCR)

The qPCR technique was achieved to find and complete quantification of microorganism deoxyribonucleic acid duplicates quantity in 45 blood samples were positive for Widal test takes a look at to ensure designation salmonellosis. These samples subjected to qPCR for confirming the designation by using SYBR Green I and flagellant gene (flic) as diagnostic gene. The results showed 21 (46.6 %) samples were positive and 24 (53.3 %) were negative (Figures 1 and 2).The results obtained by quantitative real- time polymerase chain reaction

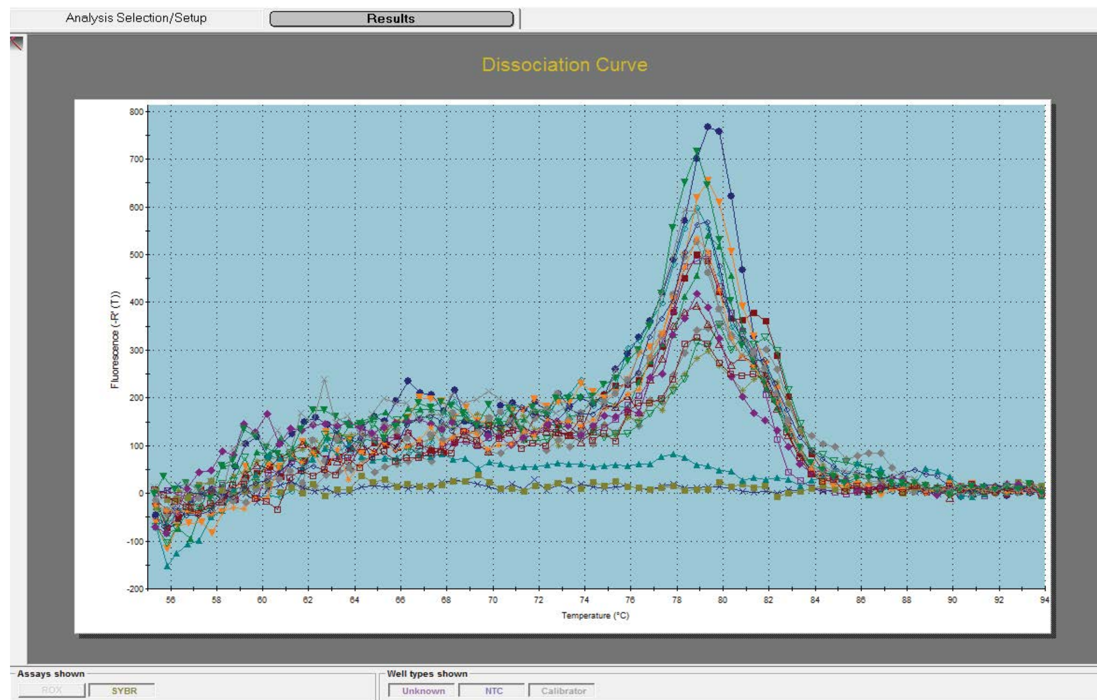

Figure 1: Dissociation curve for FliC Gene in human salmonellosis, that display a positive and negative DNA sample from human blood by using SYBER green 1 RT-PCR amplification.

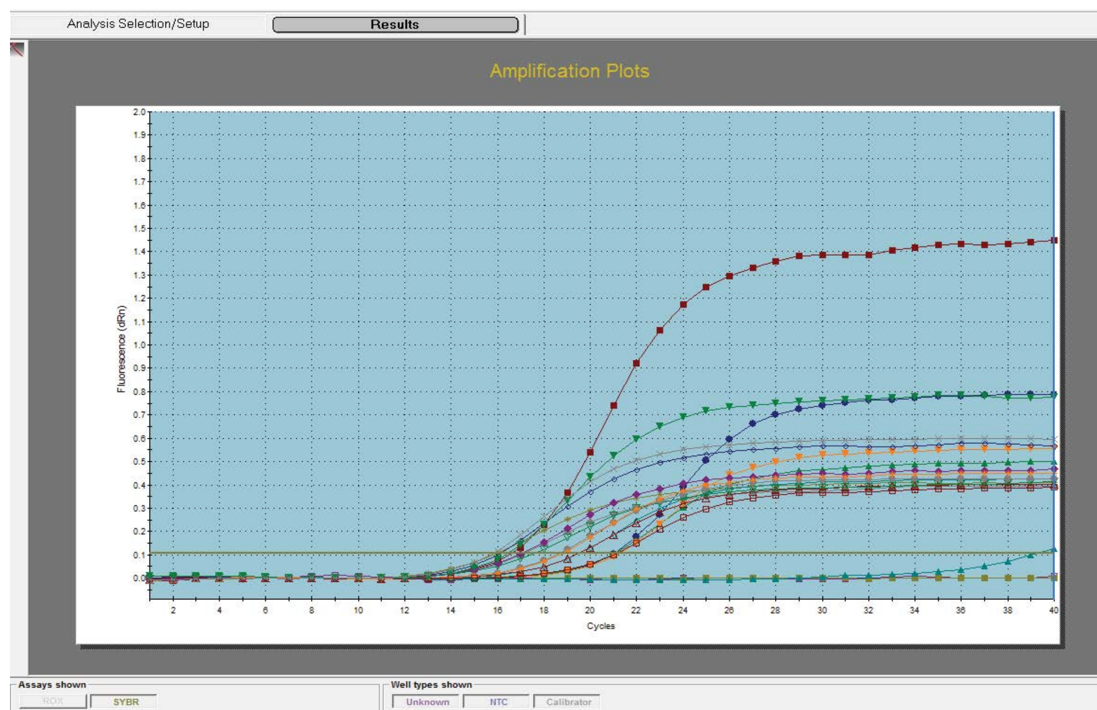

Figure 2: Real-Time PCR amplification design for FliC Gene in human salmonellosis, that display a positive and negative DNA samples from human blood, by using SYBER green 1 RT-PCR amplification.

are indicating low percentage(21%) were positive from total number 100 samples according to those result measured by Widal test (45%).The results came with an agreement with [16] results.

They observed that diagnosis of typhoid fever on clinical grounds is difficult, as the presenting symptoms are diverse and similar to those observed with other febrile illnesses [16]. The definitive diagnosis of typhoid fever requires the isolation of Salmonella Typhi or para Typhi from the patient. However, since patients often receive antibiotics prior to a laboratory diagnosis, bacteria are isolated from the blood cultures in very few cases. Besides this, the unavailability of microbiologic facilities and the long waiting time for culture results (7 to 10 days) have been identified as reasons for the preference for the Widal test. Also our results agreed with result obtained by [15] who noted that test was positive (titer to O and/or H antigen of ≥100) in 90% of patients with blood culture-positive typhoid fever and admitted during the first week of their illness. This result may reflect a population immunologically sensitized by regular subclinical exposure to S. typhi.

A high rate of false positive results in over diagnosis of typhoid fever leading to a worsening of antibiotic resistance in the country. Therefore the results of this test must be interpreted with caution taking into account the patients clinical details and history of vaccination etc. A highly specific and sensitive diagnostic test is therefore urgently required to contribute to better health in endemic resource poor settings where access to highly trained laboratory workers with adequate time is rare. One of the most advanced assays that are recently used is Real-time polymerase chain reaction as a highly sensitive and highly specific assay for detection of salmonella typhi as causative agent of salmonellosis fever. Also, due to the false positive result of Widal test or due to cross reaction result. Our result was similar to [9] who noted that the sensitivity of PCR on blood was found to be 100 per cent whereas the specificity was 76.9 per cent. The positive predictive value (PPV) of PCR was calculated to be 76.9 per cent with an accuracy of 86 per cent. Due to its high sensitivity and specificity nested PCR can be used as a useful tool to diagnose clinically suspected, culture negative cases of typhoid fever [9].

Nitr blue Tetrazolium test (NBT)

The NBT used for evaluating the phagocytic activity percentage of peripheral blood leukocytes. The results of this study show that there is a significant increase (P<0.05) in phagocytic activity in the leukocytes of patients positive Widal test, compared with deionized detailed water as a negative control (0%) and (79.3%) in the leukocytes of healthy individuals as positive control as shown in the (Table 3). The results, according to age groups indicated dissimilar noteworthy rises in the NBT index, which signifies the phagocytic activity % (-,72%, 320%, 517%, 306%, 282.7%, 200%, and 96.5%, respectively). The greatest NBT index was noted in group IV (517%), and the lowest NBT index noted in group II (72%). As shown in the (Table 3).

The innate immune system comprises many different mechanisms and components that act in a coordinated fashion to restrict an infection and eliminate the invading pathogen [15]. A diverse array of immune cells cooperates in the rapid recognition and elimination of invading microbes through phagocytosis-mediated killing and the induction of inflammation [1].

Salmonella initially interact with epithelial cells, which can recognize pathogenic bacteria and initiate an inflammatory response and recruit a variety of bone-marrow-derived phagocytes [17]. The early immune response to Salmonella in PP and MLNs involves the recruitment of neutrophils and inflammatory monocytes, and these responses are important for delaying the spread of bacteria to systemic tissues [16, 18].

Crossing the epithelial barrier allows Salmonella to escape the inhospitable environment at the surface of the intestinal mucosa and to evade many antimicrobial defenses. However, once Salmonella has passed through M cells or enterocytes, it encounters the next layer of innate immune defenses, the monocyte-derived phagocytic cells of the GALT: macrophages

Groups	Age groups/ years	Mean±SE	Phagocytic activity percentage
Negative control	-	0.029±0.001 e	0
Positive control	-	0.052±0.003 de	79.3
I	0-10	-	-
II	20-Oct	0.050±0.005 de	72
III	20-30	0.122±0.01 b	320
IV	30-40	0.179±0.01 a	517
V	40-50	0.118±0.005 b	306
VI	50-60	0.111±0.008 b	282.7
VII	60-70	0.087±0.007 c	200
VIII	70-80	0.057±0.006 d	96.5
LSD		0.0243	

Means with different letter in the same column significantly different (P<0.05)

Table 3: Nitro Blue Tetrazolium (NBT) index results according to age groups.

Groups	Age groups/ years	Mean±SE	Lymphocyte transformation index %
Negative control	-	0.082±0.005 f	0
Positive control	-	0.144±0.01 de	75.6
I	0-10	-	-
II	20-Oct	0.218±0.01 b	165.8
III	20-30	0.247±0.01 a	201.2
IV	30-40	0.224±0.01 ab	173
V	40-50	0.178±0.005 c	117
VI	50-60	0.158±0.006 cd	92
VII	60-70	0.126±0.002 e	53.6
VIII	70-80	0.085±0.004 f	3.6
LSD		0.0284	

Means with different letter in the same column significantly different (P<0.05)

Table 4: Lymphocyte transformation index for patients, according to age groups.

and dendritic cells [16]. The main function of these cells is to remove invading microbes by phagocytosis and to alert other immune cells of the infection, either directly or by secreting pro-inflammatory cytokines [16]. Following phagocytosis, Salmonellae express their virulence-associated SPI-2 T3SS to establish themselves in an intracellular compartment named the Salmonella containing vacuole (SCV) and about 90% of invader microorganism was eliminated by phagocytosis [19].

MTT assay

The MTT assay is used for evaluating the lymphocyte transformation index % of peripheral blood leukocytes in positive Widal test patients. The results of this study show that there is a significant increase (P<0.05) in lymphocyte transformation index % in the lymphocytes of patients positive Widal test, compared with deionized water as a negative control (0%) and (75.6%) in the lymphocytes of healthy individuals as positive control as it is shown in Table (4-16). According to age groups, results indicated dissimilar noteworthy rises in the lymphocyte transformation index %, (-, 165.8%, 201.2%, 173%, 117%, 92%, 53.6%, and 3.6%, respectively). The higher lymphocyte transformation index % that recorded in group III was (201.2), while the lowest lymphocyte transformation index % was recorded in group VIII (3.6) as shown in the (Table 4).

Salmonella bacteria have evolved mechanisms to evade immune defense and cause chronic infection in the host [13]. The host immune response involves innate and adaptive components that are differentially active in mucosal and systemic lymphoid tissues [17]. CD4 T cells have been shown to play a major role in protective immunity during primary and secondary Salmonella infection [20]. These CD4 T cells are activated initially in the PP and MLN after oral infection, before additional stimulation occurs in systemic tissues. In addition to CD4 T cells, innate immune cells, CD8 T cells and B cells all make an important contribution to pathogen clearance [20]. Recent studies examining no cognate stimulation of Salmonella-specific T cells suggests that expanded CD4 and CD8 effector T cells can acquire the capacity to rapidly respond to inflammatory cues, thus reducing the threshold for stimulation in infected tissues. These new findings suggest that effector T cells might be activated in a largely nonspecific manner and the development of a co-infection model may be useful to unravel this response. Future work should allow greater understanding of the induction, maintenance and stimulation of Salmonella-specific effector cells and lead to the development of improved vaccines for typhoid [21].

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